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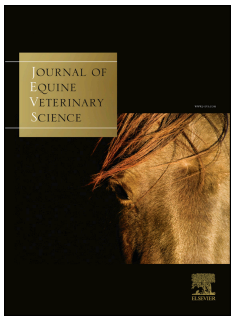


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# Pharmacokinetic Assessment of the Marker Active Metabolites 4-methyl-amino-antipyrine and 4-acetyl-amino-antipyrine after Intravenous and Intramuscular Injection of Metamizole (Dipyrone) in Healthy Donkeys

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Running Head: Pharmacokinetics of Metabolites of Metamizole in the Donkey

## ABSTRACT

Metamizole (MT) is an analgesic and antipyretic drug labelled for use in humans, horses, cattle, swine and dogs in some countries. MT is rapidly hydrolyzed to the active primary metabolite 4-methyl-amino-antipyrine (MAA). MAA is formed in much larger amounts compared to other minor metabolites. Among the other secondary metabolites, 4-amino-antipyrine (AA) is also relatively active. The aim of this research was to evaluate the pharmacokinetic profiles of MAA and AA after administration of 25 mg/kg MT by intravenous (IV) and intramuscular (IM) routes in healthy donkeys. Six jennies were randomly allocated to two equally sized treatment groups according to a 2x2 crossover study. Blood was collected at predetermined times within 24 hours and plasma was analysed by a validated HPLC UV method. Plasma concentrations of MAA after IV and IM administrations of MT were detectable from 5 minutes to 10 hours in all the donkeys. Plasma concentrations of AA were detectable from 5 minutes to 8 hours, but in smaller amounts. $C_{max}$ ($P < 0.01$), $AUC_{0\text{-}last}$, $AUC_{0\text{-}\infty}$, $AUMC_{0\text{-}last}$ and MRT ($P < 0.05$) were statistically different between the IV and IM groups. The $AUC_{IM}/AUC_{IV}$ ratio of MAA was 1.37. The AA concentrations were lower than those found for MAA. The AA plasma vs time curves profiles after the two routes of administration of MT were variable (within the groups) and different (between the groups). $T_{max}$, $\lambda z$ and $AUC_{0\text{-}last}$ were found to be statistically different between the groups ($P < 0.05$). The $AUC_{IM}$ AA/$AUC_{IV}$ AA ratio was 2.26.


***Keywords***: Analgesic; Dipyrone; Donkey; Metabolism; Pharmacokinetics

## 1. Introduction

Metamizole (sodium N-[(2,3-dimethyl-5-oxo-1-phenyl-3-pyrazolin-4-yl)-N-methylamino] methanesulphonate) (MT), also known as dipyrone, is a pyrazolone derivative [1] introduced to pharmacotherapy in 1922 in Germany [2]. This is one of the non-opioid analgesic drugs possessing highest efficacy, used in both human and veterinary medicine for the treatment of pain and fever [3]. It is a weak COX-1 and COX-2 inhibitor [4] but a strong COX-3 inhibitor [5]. Recently it has been reported that there is a potential for other likely mechanisms of action because the COX-3 inhibition may not truly explain the pharmacology of this drug [6]. MT is on the human and veterinary market in several countries (European states, Asia, and South America) but has been withdrawn in others (Sweden, USA, Japan, UK, Australia, and Iran) because of safety concerns in humans. Although MT seems to be a relatively safe drug [7,8] compared to other non-opioid analgesics there is some evidence, which is not unanimously accepted, suggesting that after prolonged administration MT might damage the haematopoietic system, triggering leukopenia, agranulocytosis and even aplastic anemia in humans [9,10,11]. However, pharmacovigilance veterinary data have indicated that the incidence of adverse reactions in the target species is very low [12]. For veterinary use, MT is administered parentally in the dose range of 20-50 mg/kg body weight (package leaflet, Biovetalgin, BioWet, Drwalew, Poland).

There is a paucity of data on the pharmacokinetic properties of MT in animals, although the fate of MT administered to humans has already been described [13]. MT is considered a prodrug which, in a hydrous environment, undergoes spontaneous breakdown to numerous metabolic products [13,14]. The parent drug is detectable in serum for just a few minutes after intravenous administration, but not after oral dosing. It is also not detectable in urine [14]. In humans, MT is rapidly hydrolyzed to the primary metabolite 4-methyl-amino-antipyrine (MAA). MAA is further metabolized to 4-formyl-amino-antipyrine (FAA), which is an end-metabolite, and to 4-aminoantipyrine (AA) [13]. AA is acetylated to 4-acetyl-amino-antipyrine (AAA) [13,14,15] (Fig.

1). MAA and AA are active metabolites [14,16]. The European Medicines Agency (EMA) dossier reports that in bovine, porcine, and equid species, MAA has been selected as a marker residue for maximum residue limit (MRL) calculation [12].

To the best of the authors' knowledge, only one report is present on the pharmacokinetics of MAA after MT intravenous administration in horses [17]. As the pharmacokinetics in horses can be different than in donkeys, the aim of the present study was to evaluate the pharmacokinetic profiles of MAA and AA after intravenous (IV) and intramuscular (IM) administrations of MT in healthy donkeys.

## 2. Material and Methods

### *2.1. Chemicals and Reagents*

Pure MAA and AA analytical standard (> 99.0% purity) were obtained from Toronto Research Chemicals (Toronto, Canada) and Sigma-Aldrich (St. Louis, MO, USA). The Internal Standard (IS) metoclopramide powder (> 99.0% purity) was supplied by Sigma-Aldrich (St. Louis, MO, USA). Donkey control plasma samples were collected in untreated healthy donkeys belonging to the same herd as the six animals selected for the treatments.

### *2.2. Animal Treatment and Sampling*

Six healthy adult Mammoth Jackstock jennies (*Equus asinus*), aged 7 to 11 years and weighing 210 to 290 kg were enrolled in the study. The jennies were determined to be clinically healthy on physical examination, serum chemistry and hematological analyses. Animals were

evaluated daily (for 1 week) for visible adverse effects by specialized personnel. Animal care and handling was performed according to the provision of the Directive 2010/63/UE (# 45/2014). Jennies were acclimatized to the stalls and handlers prior to commencing the study. Animals were deprived of food for 8 hours prior to the commencement of the experiment while water was available *ad libitum*. Hay and water were available *ad libitum* from 2 hours after treatment administration.

Animals were randomly allocated to two treatment groups (A=3 and B=3) (six slips of paper marked with the numbers 1 to 6 in a box) according to an open, single-dose, two-treatment and two-period crossover design experiment. Two jugular venous catheters, one in each side (for MT administration and for sample collection, respectively), were placed in each animal 1 day prior to commencement of the study. The group A animals received a single dose of MT (25 mg/kg) by intravenous injection (IV) (Biovetalgin, injectable solution 500 mg/mL, BioWet, Drwalew, Poland) while the group B animals received MT at the same dose by intramuscular injection (IM), injected in the middle quadrant of the neck muscle (Biovetalgin, injectable solution 500 mg/mL, BioWet, Drwalew, Poland). The dose was selected based on package leaflet recommendations for equid species. An interval of 1 week (wash-out period) was observed to ensure complete metabolism and excretion of MAA and AA. After this period, the groups were rotated and the crossover study completed. By the end of the study, each donkey had received MT by both administration routes. The blood (3 to 5 mL) was collected via previously inserted catheters at assigned times (0, 15, 30, 45 minutes and 1, 1.5, 2, 4, 6, 8, 10 and 24 hours). The samples were centrifuged at 1,000 x *g* within 30 minutes of collection and the harvested plasma was frozen immediately and stored at -20° C. Samples were analysed within 1 week of collection.

*2.3. HPLC-FL*

The analytical method was based on a previously described method [18] with slight modifications [19]. The HPLC system was an LC Jasco (Como, Italy) that consisted of a quaternary gradient system (PU 2089 PLUS), in line with an ultraviolet detector (Jasco UV-975) set at 254 nm. The chromatographic separation assay was performed with a Luna C18(2) analytical column (250 mm × 4.6 mm inner diameter, 5 µ particle size [Phenomenex, Bologna, Italy]) preceded by a security guard column with the same stationary phase (C18(2) [Phenomenex, Bologna, Italy]). The system was maintained at 25°C. The mobile phase consisted of acetonitrile:amonium acetate (20 m*M*) solution, pH 5 (20:80, v/v) at a flow rate of 1 mL/min. The elution of the substances was carried out in isocratic mode.

*2.4. Sample Extraction*

The procedure was performed in a 15 mL polypropylene vial. A 0.5 mL aliquot of plasma was added to 100 µL of IS (25 µg/mL). After 30 seconds vortexing, 0.1 mL sodium hydroxide (1 N) was added and the sample vortexed again. An aliquot of 4 mL of ethylacetate: methylene chloride (3:7, v/v) was added, then vortexed (30 seconds), shaken (60 osc/minute, 10 minutes) and centrifuged at 10,956 x *g* (rotor radius 5 cm) for 10 minutes at 10°C. Three mL of supernatant was collected in a new 15 mL screw cap vial. The organic phase was evaporated under a gentle stream of nitrogen (40°C) and reconstituted with 100 µL of mobile phase. Fifty µL of this solution was injected onto the HPLC.

*2.5. Pharmacokinetic Analysis and Statistical Analysis*

The pharmacokinetic calculations were carried out using WinNonlin v 5.3.1 (Pharsight Corp). The curve fit was performed by a non-compartmental analysis. The pharmacokinetic parameters are presented as geometric mean and pseudo SD.

In order to make comparisons across treatments, the different parameters were first tested for normal distribution and variance homogeneity. Data were compared with the paired t-test or the non-parametric Wilcoxon test, depending on whether the data passed a normality test. In all experiments, differences were considered significant if $P < 0.05$.

## 3. Results

The HPLC method was revalidated using control plasma from donkey. Briefly, MAA and AA were linear in the range of 100–10000 ng/mL. LOD was 30 ng/mL and LOQ was 50 ng/mL, respectively. When the metabolite concentrations in the samples exceeded the upper limit of the range, they were re-analysed after appropriate dilution. The intra-day repeatability was lower than 5.2 and 5.9%, whereas accuracy was lower than 4.4 and 5.9% for MAA and AA, respectively. No behavioural changes or alterations in health parameters were observed in the IV or IM groups of animals during or after (up to 7 days) the drug administration.

Both the injections (IV and IM) where well tolerated without any discomfort sign, pain or swelling shown from the animals. Plasma concentrations of MAA after IV administration of MT were detectable from 5 minutes to 10 hours in all donkeys of both administration groups. The $C_{max}$ of MAA was higher in the IV than in IM group and this concentration was achieved earlier in the IV group (0.08 vs 0.87 hours). Half an hour after MT injection, the average MAA plasma concentrations of IV and IM groups became similar. From 1 to 6 hours, MAA plasma concentrations were higher in the IM than in the IV group ($P<0.05$). The percent of AUC that was extrapolated to infinity was always <20% in all the subjects. The average pharmacokinetic curves

are shown in Fig. 2. The main pharmacokinetic parameters are reported in Table 1. $C_{max}$ ($P < 0.01$), $AUC_{0\text{-last}}$, $AUC_{0\text{-}\infty}$, $AUMC_{0\text{-last}}$ and MRT ($P < 0.05$) were statistically different between the groups. The mean $AUC_{IM\ MAA}/AUC_{IV\ MAA}$ ratio was 1.37.

Plasma concentrations of AA after IV administration of MT were detectable from 5 minutes to 8 hours in all donkeys of both administration groups. These concentrations were lower than those found for MAA. The average pharmacokinetic profiles of AA are reported in Fig. 3. The AA plasma vs time curves profiles after the two routes of administration of MT were variable (within the groups) and different (between the groups). The $AUC_{IV\ MAA}/AUC_{IV\ AA}$ ratio was 23.5 while the $AUC_{IM\ MAA}/AUC_{IM\ AA}$ ratio was 14.2. The main pharmacokinetic parameters are reported in Table 2. $T_{max}$, $\lambda_z$ and $AUC_{0\text{-last}}$, were parameters found to be statistically different between the groups ($P < 0.05$). The $AUC_{IM\ AA}/AUC_{IV\ AA}$ ratio was 2.26.

## 4. Discussion

Metamizole, a nonnarcotic analgesic, has been used to treat pain and fever for almost 90 years in some countries, while in others it is completely unknown or forgotten [20]. MT is known to possess high pain-relieving activity, antipyretic and spasmolytic properties [13] and does not have the contraindications or limitations usually observed with opioids or NSAIDs [3,21,22,23,24]. MT has been shown to be a safe and important drug for the management of pain but its use in humans is still controversial. There is plenty of literature attesting to the analgesic efficacy of MT in human beings [21,22,23,24,25,26].

In veterinary medicine however, the scenario is totally different since the evidence from veterinary studies is not as strong as that from the human literature. There are some data available concerning clinical and side effects of MT in horses [27], rabbits [3], rats [28], and dogs [8,29,30,31] and some concerning the pharmacokinetic profile of its metabolite MAA in horses [17,], rats and dogs [32], swine [33] and sheep [19].

MT is a drug labelled for use in horses, cattle, swine, dogs and cats. In the last few years, thanks to its attractive pharmacological features, safety profile and low price, there has been a rising interest in the use of MT in the veterinary field. However, pharmacokinetic-pharmacodynamic data in donkeys are absent from the literature. A single study [17] reporting the pharmacokinetics of MAA after a single IV administration of MT in horses is present in the literature. However, the differences in drug dispositions between horse and donkey are well known and documented in literature [34,35,36,37,38, 39,40] and the data extrapolations are risky. A species specific study is needed to evaluate the pharmacokinetics of MT in donkeys.

The overall pharmacokinetic profiles of MAA after IM and IV administrations of MT were similar. The significant differences found in $C_{max}$ values were ascribable to the routes of administration of MT. The complete/immediate introduction of MT into the vascular compartment (IV injection) may have generated, in the initial minutes, a more rapid metabolic conversion (increasing the $C_{max}$ of MAA) compared to the IM injection where an absorption phase is expected. The absorption phase may also be responsible for the difference in $T_{max}$. The larger AUCs, $t_{1/2}$ $\lambda z$ and MRT values found in the IM group are likely due to the gradual release of drug from the injection site to the vascular system. Despite the abrupt peak of MAA concentration following IV administration, no adverse effects were shown in the animals.

The plasma concentrations of MAA detected in the present study were higher than those previously observed in horses [17], administered with the same dose of MT. However, the drug administered in the horse study [17] was a combination product labelled for humans which also contained hyoscine butylbromide (Buscopan compositum, Boehringer-Ingelheim, Ingelheim, Germany). Pharmacokinetic interactions between the two active compounds might have affected the MT metabolism or the MAA kinetics. However, the $AUC_{0-\infty}$ values of MAA are comparable between the studies.

The half-life reported in the present study was shorter than those previously reported in dogs (4-5 hours; [41]) and horses (4.85 hours; [17]) but similar to that reported in sheep (1.45-3 hours;

[19]). The reason for this difference might be due to a number of factors such as: differences in animal species, route of administration, presence of pathophysiological conditions, age of the animals, and sensitivity of the analytical method. Volume of distribution (assuming that all MT is transformed into MAA) is large, a finding in line with the chemical/physical features of MAA, for example it is detected in cerebrospinal fluid [42].

Diverse pharmacokinetic trends have been found for the AA metabolite after IM and IV administrations of MT. As a result, the $AUC_{0\text{-last}}$ value of the IV group was half that found in the IM group. The abrupt peak of MAA concentration following IV administration might have saturated the metabolic pathway MAA to AA, metabolizing (oxidizing) a proportion of MAA to FAA (inactive metabolite). This might explain why the $C_{max}$ values of AA between the groups are not statistically different. Further studies evaluating all the metabolites formed in the donkey are necessary to clarify this issue.

In humans the analgesic effect of MT correlates with the concentration of MAA and AA, which differ with regard to their time of onset (MAA > AA) and terminal half-life (MAA: 4-5 hours, AA: 5–8 hours) [20]. MAA is around 50 times more active than MT as an inhibitor of COX, while AA is less active than MT. Therefore both metabolites can contribute to the clinically relevant features of rapid onset and long duration of the effect, permitting 8–10 hourly dosing intervals. The other 2 metabolites, FAA and AAA, are inactive. The metabolites which generate the analgesic action, are still unknown in the donkey. According to the drug producer, a 25 mg/kg injection of MT is an effective dose to relieve pain in equid species for 10 hours. If we assume that analgesic activity in the donkey is only attributable to MAA and AA metabolites as in humans, the contribution of AA to the overall therapeutic activity might be negligible due to its negligible plasma concentrations and activity. Hence, it might be presumed that MAA is the main metabolite responsible for the effect. A gross calculation of the average plasma concentration of this metabolite (which is likely to produce pain relief in the donkey), in other words its minimal effective concentration, might theoretically be calculated as $AUC_{0\text{-last}}$/10 hours and approximated to be above

8 µg/mL. Further studies are necessary to confirm this theoretical data and to establish if the metabolic pattern reported in humans matches that in donkeys, as well as pharmacodynamic studies to determine its efficacy in different types of pain.

## 5. Conclusions

This is the first study reporting the pharmacokinetics of MAA and AA after IV and IM administration of MT in donkeys. The IV administration of MT elicits MAA plasma concentration lower than that after IM administration, while twice the amount of AA is formed after IM administration of MT. Although further studies are needed to understand the metabolic pathway of MT as well as its safety profile, the difference reported in AA concentrations might be clinically negligible in donkeys.

## Conflict of Interest Statement

None of the authors has any financial or personal relationships that could inappropriately influence or bias the content of the paper.

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**Figures' captions**

**Fig. 1.** Metabolic pathway of metamizole (MT) reported in humans [41].

**Fig. 2.** Mean plasma concentrations of 4-methyl-amino-antipyrine (MAA) vs. time curves following intravenous (—●—) and intramuscular (--○--) administrations of 25 mg⁄kg metamizole (MT) in healthy jennies ($n$ = 6). Bars represent the standard deviations. * Statistically different between the groups.

**Fig. 3.** Mean plasma concentrations of 4-aminoantypyrine (AA) vs. time curves following intravenous (—●—) and intramuscular (--○--) administrations of 25 mg⁄kg metamizole (MT) in healthy jennies ($n$ = 6). Bars represent the standard deviations. * Statistically different between the groups.

Table 1

**Table 1**
Main pharmacokinetic parameters of 4-methyl-amino-antipyrine (MAA) following single intravenous (IV) and intramuscular (IM) administrations of metamizole (MT) (25 mg/kg) in healthy jennies (n=6).

| | IV | | | IM | | |
|---|---|---|---|---|---|---|
| Parameter | Mean | | SD | Mean | | SD |
| $R^2$ | 0.98 | ± | 0.01 | 0.94 | ± | 0.04 |
| λz (1/h) | 0.40 | ± | 0.10 | 0.35 | ± | 0.10 |
| $t_{1/2}$ λz (h) | 1.81 | ± | 0.49 | 1.94 | ± | 0.79 |
| $T_{max}$ (h) | 0.08 | ± | 0.00 | 0.87 | ± | 0.12 |
| $C_{max}$ (µg/mL)* | 211.72 | ± | 21.58 | 46.33 | ± | 13.23 |
| $AUC_{0\text{-}last}$ (h µg/mL) * | 82.92 | ± | 5.46 | 113.44 | ± | 45.02 |
| $AUC_{0\text{-}\infty}$ (h µg/mL) * | 83.01 | ± | 5.46 | 120.33 | ± | 55.12 |
| Vz/F (mL/kg) | 786.23 | ± | 221.33 | 503.01 | ± | 389.67 |
| Cl/F (mL/h/kg) | 301.42 | ± | 20.01 | 161.58 | ± | 195.42 |
| $AUMC_{0\text{-}\infty}$ ($h^2$ µg/mL)* | 92.23 | ± | 14.67 | 273.98 | ± | 112.41 |
| MRT (h) * | 1.04 | ± | 0.13 | 2.99 | ± | 1.04 |

$R^2$ = correlation coefficient; λz = terminal phase rate constant; $t_{1/2}$λz = terminal half-life; $T_{max}$ = time of peak; $C_{max}$ = peak plasma concentration; $AUC_{0–last}$= area under the plasma concentration-time curve; $AUC_{0\text{-}\infty}$= area under the plasma concentration-time curve extrapolated to infinity; Vz/F = apparent volume of distribution; Cl/F = apparent clearance; $AUMC_{0\text{-}\infty}$= area under the first moment curve from zero to infinity; MRT = mean resident time.
Parameters values are expressed as geometric mean, while $t_{1/2}$ λz as harmonic mean. PSD = pseudo standard deviation. * Statistically different value between the treatment groups.

407 Table 2

408

**Table 2**

Main pharmacokinetic parameters of 4-amino-antipyrine (AA) following single intravenous (IV) and intramuscular (IM) administrations of metamizole (MT) (25 mg/kg) in healthy jennies (n=6).

| | IV | | | IM | | |
|---|---|---|---|---|---|---|
| Parameter | Mean | | PSD | Mean | | PSD |
| $R^2$ | 0.90 | ± | 0.15 | 0.97 | ± | 0.01 |
| λz (1/h)* | 0.17 | ± | 0.02 | 0.26 | ± | 0.06 |
| $t_{1/2}$ λz (h) | 3.87 | ± | 0.56 | 2.68 | ± | 0.94 |
| $T_{max}$ (h)* | 0.16 | ± | 0.09 | 0.94 | ± | 0.23 |
| $C_{max}$ (µg/mL) | 2.82 | ± | 2.13 | 2.36 | ± | 0.85 |
| AUC $_{0\text{-last}}$ (h µg/mL)* | 3.52 | ± | 0.81 | 7.98 | ± | 2.13 |
| MRT (h) | 4.58 | ± | 0.81 | 4.56 | ± | 1.06 |

$R^2$ = correlation coefficient; λz = terminal phase rate constant; $t_{1/2}$λz = terminal half-life; $T_{max}$ = time of peak; $C_{max}$ = peak plasma concentration; $AUC_{0–last}$= area under the plasma concentration-time curve; MRT = mean resident time.

Parameters values are expressed as geometric mean, while $t_{1/2}$ λz as harmonic mean. PSD = pseudo standard deviation. * Statistically different value between the treatment groups.

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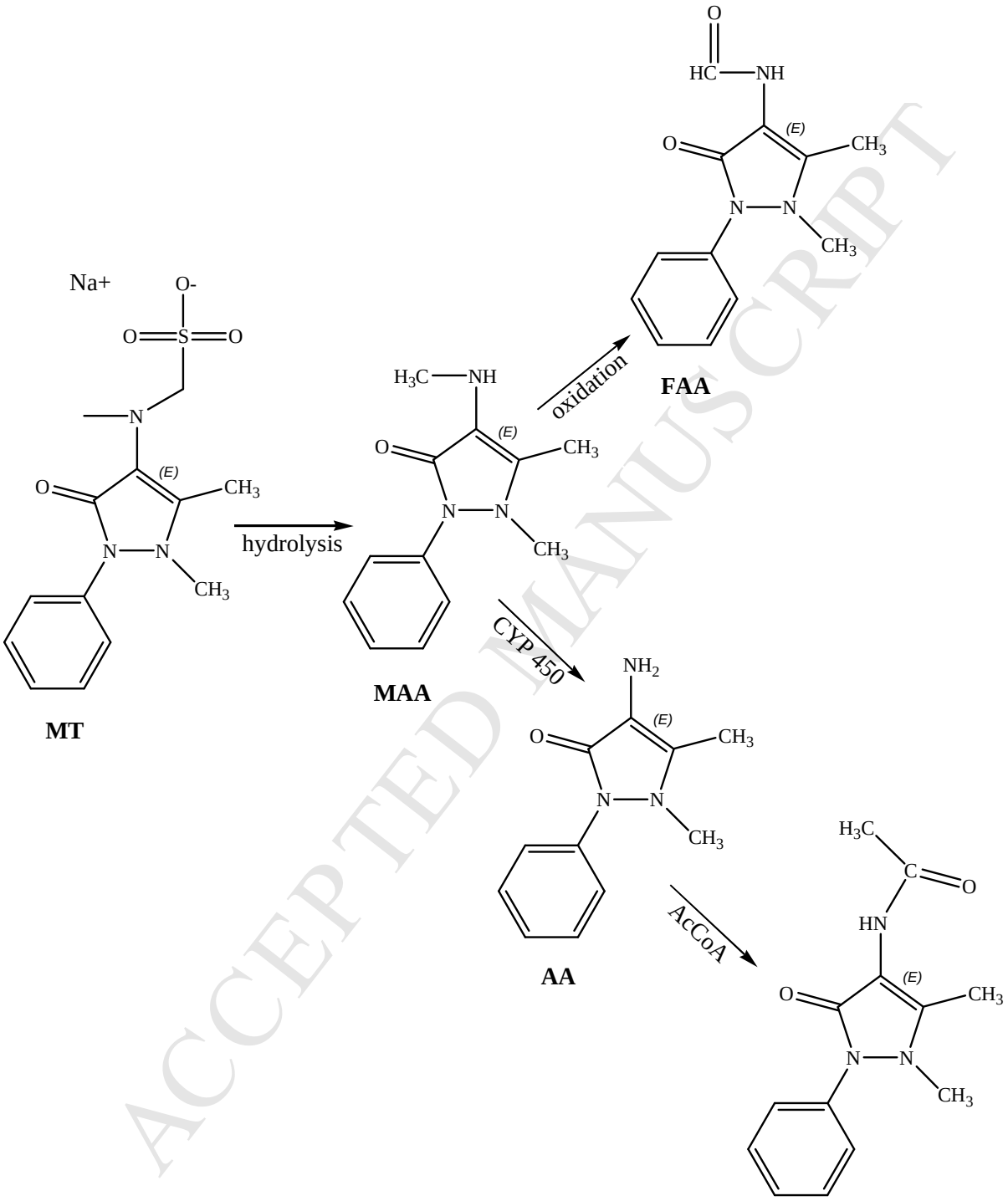
411 Fig. 1.

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414 Fig. 2.

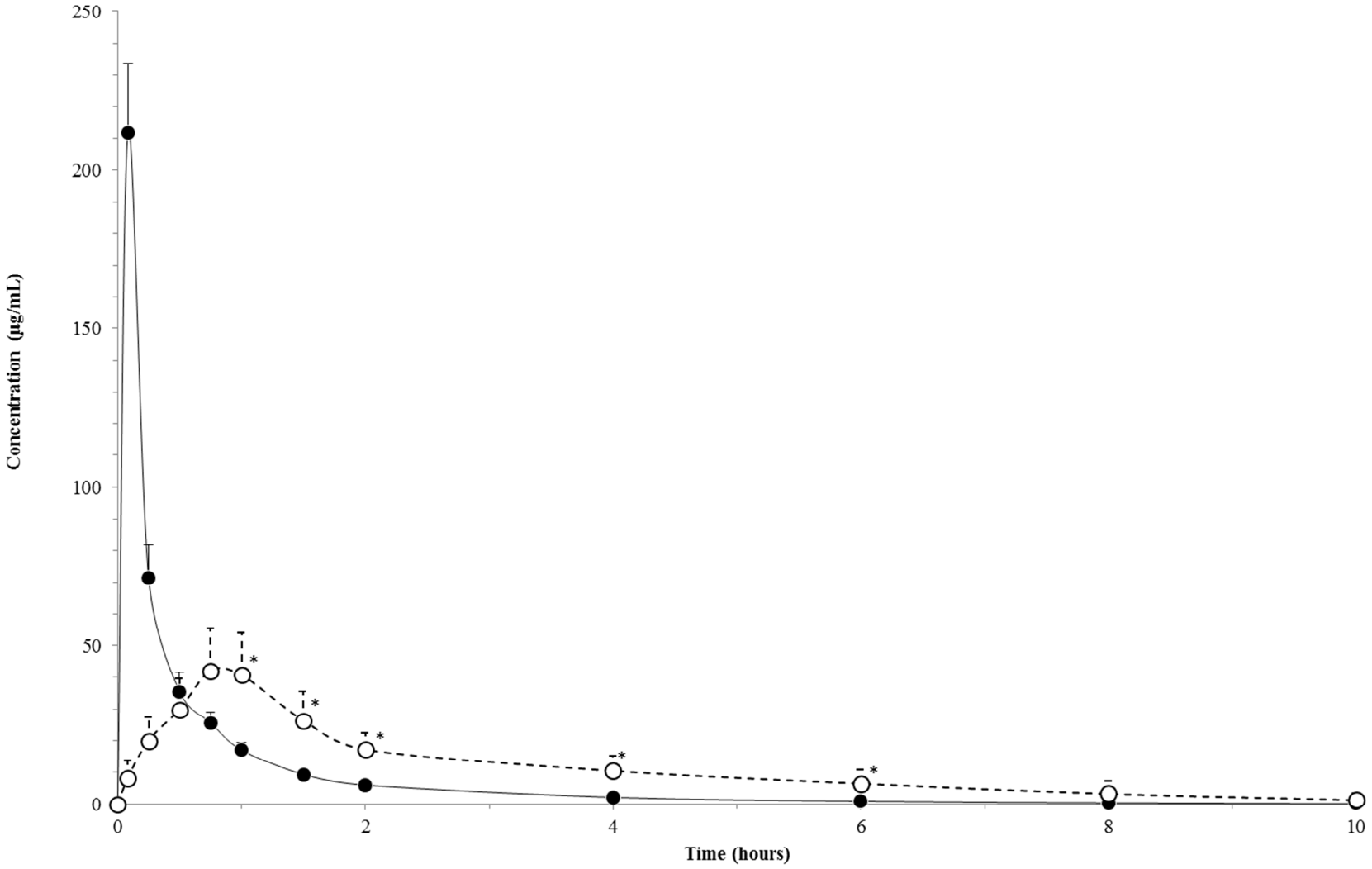


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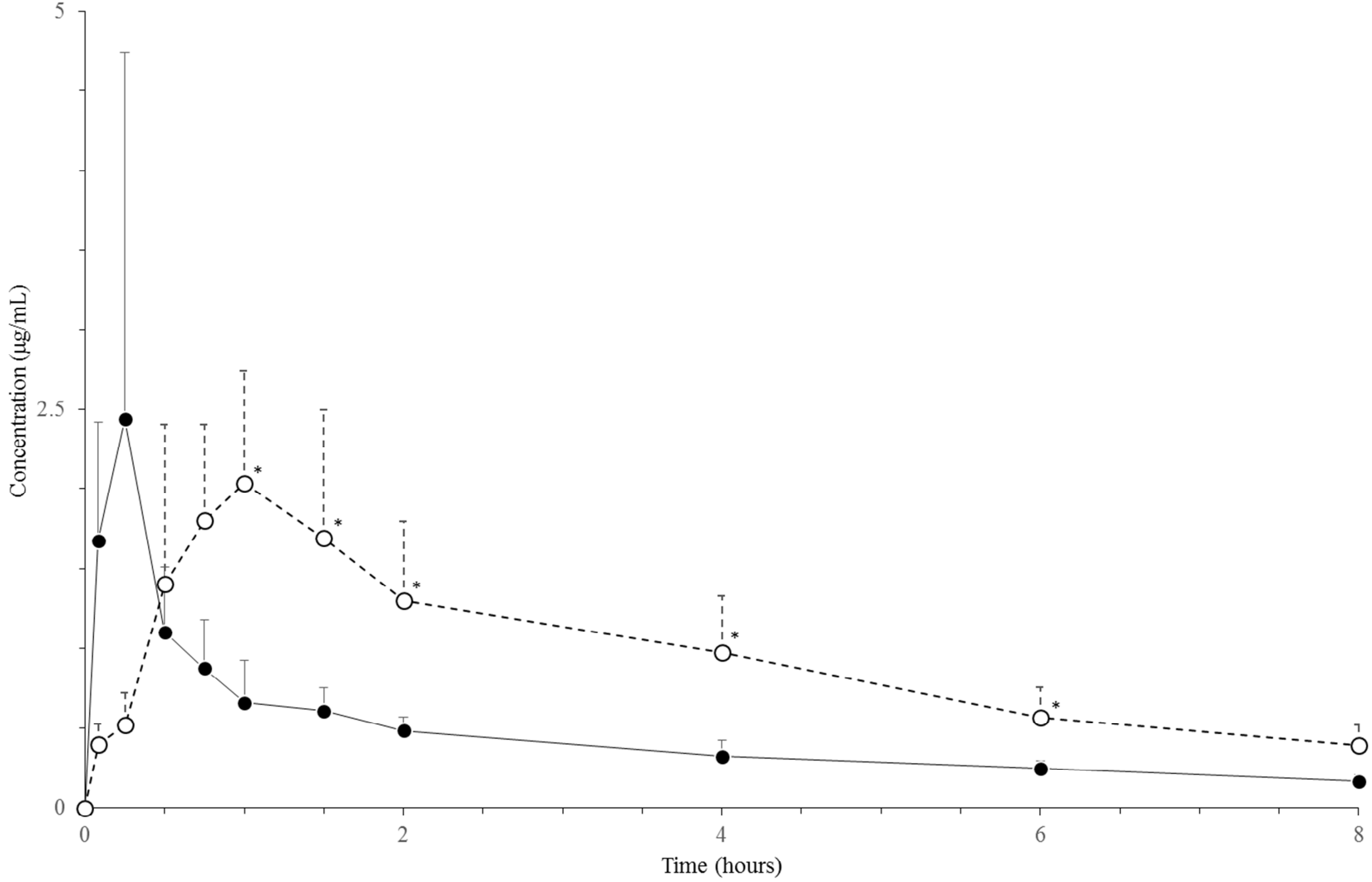
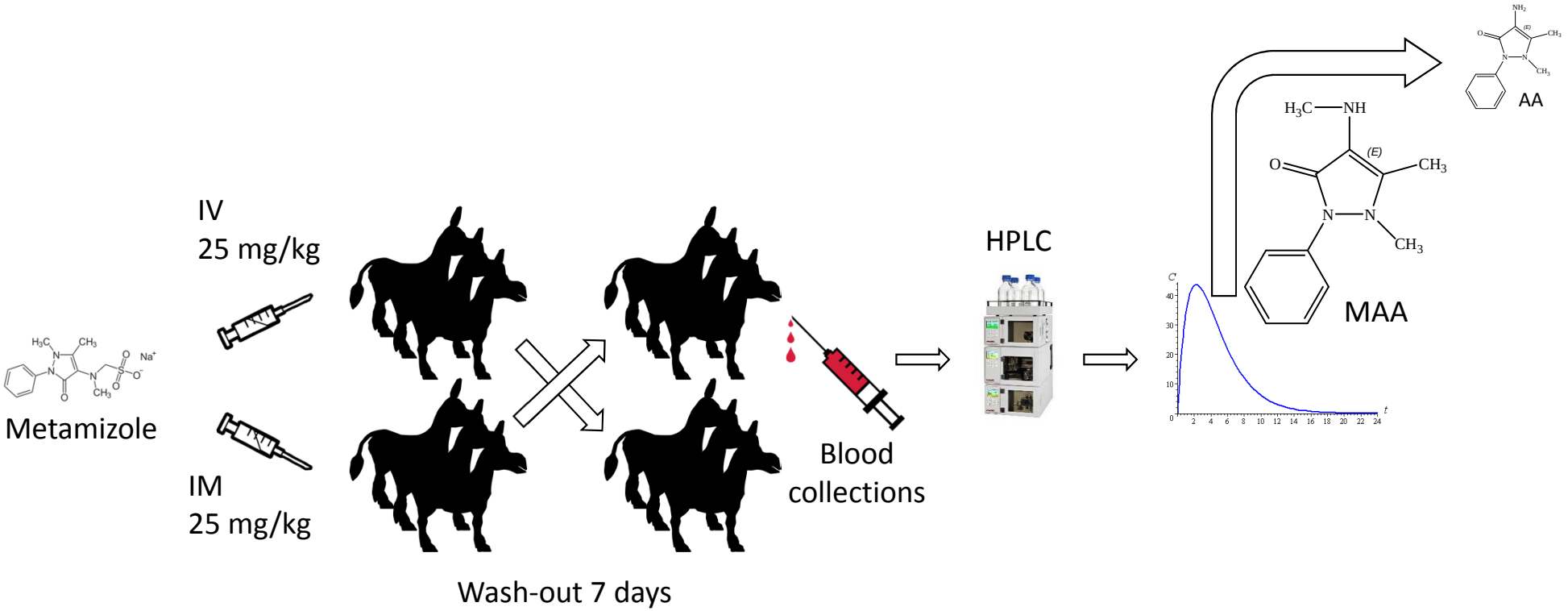
Fig. 3.




Metamizole
IV
25 mg/kg
IM
25 mg/kg
Wash-out 7 days
Blood
collections
HPLC
MAA
AA

Highlights

Metamizole is metabolized in vivo in donkeys

4-methylamino-antipyrine is produced in larger amounts than 4-acetylamino-antipyrine

After IM and IV administration of metamizole, the plasma profiles of the metabolites are different